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High-performance liquid chromatographic–electrospray mass spectrometric analysis of bile acids in biological fluids

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Abstract

The present work describes the development of HPLC–mass spectrometric systems equipped with an electrospray interface for the quantitative analysis of bile acids. Good separation of free as well as glycine- and taurine-conjugated bile acids was achieved with a C_{18} reversed-phase column (3 μm particle size, 70 × 4.6 mm I.D.) employing methanol–15 m*M* ammonium acetate as the mobile phase for both isocratic and gradient mode, at a flow-rate of 0.3 ml/min. This system permits post-column splitting of the eluate for analysis by two different detectors: (1) electrospray-mass spectrometer with a flow-rate of 18 μl/min; and (2) a complementary evaporative light scattering mass detector. When bile salts were ionized in the electrospray interface operating in the negative-ion mode, only $[M-H]^-$ molecular ions were generated; the detection limit was 15 pg injected for all bile acids studied. In the second system, a semi-micro pre-column splitting apparatus (Acurate, LC Packings) was utilized: with this device the flow-rate from the HPLC pump was reduced to 1.4 μl/min and bile acids were separated with a micro-bore C_{18} column (3 μm particle size, 150 × 0.30 I.D.), using the same mobile phase as above. With this latter system, a head-column enrichment technique can be used: the amount injected can be increased from 60 to 200 nl, permitting an improvement in the detection limit to 5 pg injected. Application of the HPLC–electrospray-mass spectrometric method to bile and serum bile acid analysis is described; preliminary data on the ability of the first system to determine the $^{13}C/^{12}C$ isotope ratio in ^{13}C-labeled bile acid enriched serum is also critically discussed.

1. Introduction

Bile is a complex mixture of substances including a wide variety of naturally occurring bile acids (BAs). These BAs are categorized by class, each of which is characterized by a particular substituent on the steroid nucleus; in addition, the side chain of the BA molecule can be conjugated with glycine, taurine or, less frequently, other molecules. In the enterohepatic circulation, BA undergo a series of metabolic steps at both the intestinal and hepatic level, making identification and comprehensive study of their metabolic profiles in this system quite difficult. BAs also differ among the various animal species and, within a given species, the qualitative composition of bile can differ as a consequence of hepato-biliary diseases.

Separation and eventual identification of BA are currently achieved by chromatographic tech-

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niques that are more or less laborious. HPLC with UV detection [1–9], and more recently, HPLC with evaporative light scattering mass detection [10–13] are used for simple analysis in liquids containing millimolar quantities of BA, whereas GC–MS or HPLC–MS are utilized for less concentrated fluids. GC–MS is widely employed for identifying many BAs in various metabolic stages, but this technique entails a series of laborious pre-analytical steps, which limits its analytical performance considerably [14–18]. Indeed, the determination of the free, glycine- and taurine-conjugated forms present in a given matrix requires preliminary separation of the BAs by class and several derivatization procedures. Recently, Setchell and Vestal [19] developed an HPLC–thermospray MS method for the direct analysis of BAs in biological matrices such as bile; although only the analysis of glycine- and taurine-conjugated BA was reported, these authors showed this method to be moderately sensitive. Technological advances in interfacing HPLC–MS have led to the development of electrospray systems (ES) [20–22] which turned out to be extremely useful for the analysis of proteins and other high-molecular-mass substances [23–27]. Because this technique also seemed to have good potential for BA analysis, we developed an HPLC–MS method with an electrospray interface for the qualitative and quantitative analysis of BA in biological fluids. Our intention was also to set up a system that would be directly applicable to the matrix, and capable of separation and identification of all BAs present (free, glycine- and taurine-conjugated forms), in a single chromatographic run, with high sensitivity. The potential use of this system for pharmacokinetic studies using BA labeled with the stable carbon isotope ^{13}C is also reported.

2. Experimental

2.1. Chemicals

All chemicals and solvents used were of analytical grade. HPLC-grade methanol, acetic acid and ammonium hydroxide were purchased from Farmitalia, Carlo Erba (Milan, Italy). Glycine and taurine amidated and unconjugated bile acids were purchased from Sigma (St. Louis, MO, USA). Ursodeoxycholic acid (UDCA) was supplied by Giuliani (Milan, Italy). The following BA standards were used: tauroursodeoxycholic acid (TUDCA), glycoursodeoxycholic acid (GUDCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), glycocholic acid (GCA), cholic acid (CA), taurochenodeoxycholic acid (TCDCA), glycochenodeoxycholic acid (GCDCA), chenodeoxycholic acid (CDCA), taurodeoxycholic acid (TDCA), glycodeoxycholic acid (GDCA), deoxycholic acid (DCA), glycolithocholic acid (GLCA), taurolithocholic acid (TLCA), and lithocholic acid (LCA).

^{13}C-Labeled glycoursodeoxycholic acid was synthesized in our laboratory using the mixed anydride method form UDCA and [1-^{13}C]-glycine supplied by Tracer Technologies (Sommerville, MA, USA).

2.2. Instrumentation

BAs were analyzed with an HPLC–MS system equipped with an electrospray interface (ES-MS, Fisons Instruments, Altrincham, UK). The apparatus consisted of a 600E Multisolvent Waters pump (Waters, Milford, MA, USA) connected with an autosampler injector (Waters 717). A part of the eluate from the column (Ultrasphere XL C_{18}, 3 μm particle silica size, 70 × 4.6 mm I.D.; Beckman Instruments, Berkeley, CA, USA) was split off on-line into the electrospray interface and quadrupole mass spectrometer (VG TRIO 2000, Fisons Instruments). The remaining eluate was routed into an ELSD II evaporative light scattering mass detector (Varex Corporation, Burtonsville, MD, USA) as a complementary means of analysis; the signal of this detector was recorded by means of a Waters 746 data module (Fig. 1a). We also utilized a second system, a semi-micro apparatus consisting of an Acurate system (LC Packings, Zurich, Switzerland) in which the flow-rate of the mobile phase is reduced from 0.3 ml/min to 1.4 μl/min, directly after the 600 MS pump. The sample

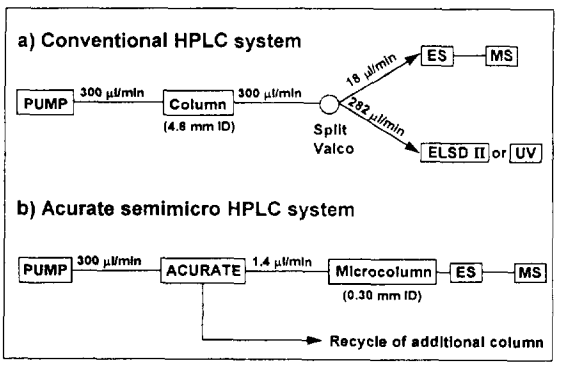

Fig. 1. Schematic representation of the electrospray interface coupled with: (a) the conventional HPLC system, (b) a semi-micro column using the Acurate system.

(60–200 nl) was injected with a Valco microinjector (Valco, Houston, TX, USA) connected to a C_{18} Fusica 3-μm particle size, 15 cm × 300 μm I.D. column (LC Packings). The mobile phase was the same as that used for the conventional system (Fig. 1b).

2.3. Analytical conditions

Bile acid extraction

Bile acids are present in biological fluids such as serum or urine at very low concentrations; in serum, many are tightly bound to protein. For analysis of BAs, the complexity of these biological fluids requires a pre-analytical step in which the BAs are selectively isolated from the other components.

Common BAs are usually isolated from biological fluids by means of reversed-phase C_{18} column extraction, as described by Setchell and Worthington [28]. The sample (100–200 μl) is diluted (1:4, v/v) with 0.1 *M* NaOH and added to the column (C_{18} Bond Elut, Analytichem International, Harbor City, CA, USA) that is previously activated with methanol and water. The column is washed with water, the methanol eluate is dried, and the residue dissolved with a suitable quantity of mobile phase, depending upon the expected BA concentration, for later HPLC separation. Serum requires an additional pretreatment: usually 0.1–0.5 ml of serum is diluted with 3.5 ml of 0.1 *M* NaOH and incubated in a water bath for 30 min at 64°C.

Combining a C_{18} column with a Bond Elut SAX (Analytichem International) anion-exchange column, good separation of the three BA groups (free, tauro- and glyco-conjugates) is achieved, as has been described by Scalia [29].

Conventional HPLC

For the separation of glyco- and tauro-conjugated BA, a methanol–15 m*M* ammonium acetate solution (66:44, v/v) with an apparent pH 5.4 ± 0.1 was utilized (solvent A); the conditions were isocratic at a flow-rate of 0.3 ml/min.

Free BAs were separated and eluted, within an acceptable time limit, with methanol–15 m*M* ammonium acetate solution (80:20, v/v) having an apparent pH of 6.0 ± 0.1 (solvent B); this too was carried out under isocratic conditions at a flow-rate of 0.3 ml/min.

We also developed a gradient system for the simultaneous separation of all BAs, free as well as those conjugated with glycine and taurine, within a single run. This consisted of a ternary gradient in six steps (Table 1) employing solvent A (66:34, v/v, methanol–15 m*M*, pH 5.38 ammonium acetate), solvent B (75:25, v/v, methanol 15 m*M*, pH 6.0–ammonium acetate), and solvent C (100% methanol). The flow-rate in this system was 0.3 ml/min at the outlet of the column.

Because the electrospray interface requires flow-rates not higher than 10–20 μl/min, it is

Table 1
Mobile phase gradient composition used for the simultaneous separation of free, lycine- and taurine-conjugated bile acids

Time (min)	Solv. A (%)	Solv. B (%)	Solv. C (%)
Initial	90	–	10
15	90	–	10
23	100	–	–
40	–	80	20
50	–	100	–
60	–	65	35
70	90	–	10

The compositions of the three solvent systems are reported in the text.

necessary to split off part of the solvent, at a fixed and constant ratio. This can be done by connecting the column to a T-shaped Valcro HPLC splitter; this fixture is connected to the column outlet where the flow-rate is 0.3 ml/min. One arm of the T is connected to a narrow-bore PTFE tube from which the excess mobile phase flows and can be recycled, or the flow can be routed into a conventional complementary detector (UV) or, as in the present study, into an ELSD II. The other arm (length: 30 cm, I.D.: 62 μm) is connected to the probe of the ES interface system in which the flow-rate at the electrospray interface is 18 μl/min.

Semi-micro HPLC

As an alternative, a semi-micro HPLC system can be used; this system eliminates the post-column split; it couples the HPLC microcolumn outflow directly to the electrospray interface, with a flow-rate of 1.4 μl/min.

The sample was injected with loops of 60 and 200 nl; with the latter, the detection limit was further improved since, with this particular loop, the analyte could be dissolved in methanol–water (20:80, v/v), resulting in head-column enrichment.

2.4. Bile acid analysis in biological fluids

Different biological samples were analyzed by HPLC–ES-MS to verify the analytical performance of the developed system on real samples. When the detection limit was compatible, as in the case of bile analysis, the ES-MS qualitative and quantitative data were compared with those obtained with the ELSD II connected on line.

- Gallbladder bile samples of hamster chronically treated with 6-fluoro-3α,7β-dihydroxy-5β-cholanoic acid, a synthetic analog of UDCA were analyzed.
- Serum samples from normal hamster and human patients with liver disease, with total BA concentrations ranging from 0.1 to 100 μmol/l were analyzed.
- Analysis of ^{13}C-labeled GUDCA in serum and calculation of the $^{13}C/^{12}C$ isotope ratio for pharmacokinetic studies was also performed. The C^{13}/C^{12} isotope ratio was assessed on the eluted GUDCA peak using the highest resolution possibly with the MS used.

3. Results and discussion

3.1. Electrospray MS interface optimization

The individual BA were analyzed as anions in the negative-ion acquisition mode (ES^-). The pK_a values of unconjugated ($pK_a = 5$) and glycine-conjugated ($pK_a = 3.9$) BA require the use of a mobile phase with an apparent pH of at least one unit higher than the corresponding pK_a to ensure complete ionization. In the case of taurine-conjugated BA ($pK_a \cong 1$), the pH was not relevant in determining the validity of the method.

The best response of the ES interface was obtained using purified and filtered air instead of nitrogen because the oxygen in air, by acting as an electron scavenger, presents undesirable charging.

The Fisons ES was optimized by direct loop injection of the individual BA. The lower detection limit (at signal-to-noise ratio of 3), 10 pg injected, was obtained under the following conditions: probe voltage, 3.19 kV; counter electrode, 0.54 V; cone voltage, 56 V; the source temperature, 68°C. The optimum flow-rate of the mobile phase was 18 μl/min. Under the above conditions, the MS spectra obtained were those of the intact negative ions, not fragmented ions or adducts. A typical ES–MS spectrum of GUDCA obtained with total-ion counting is shown in Fig. 2. The detector response was similar for all analyzed BAs.

3.2. Conventional HPLC analysis

In this study chromatographic separation of BA was achieved under conditions similar to those previously reported [10]. In a previous study, we used a column with similar I.D. and stationary C_{18} package, but the column length was much longer (25 cm *vs.* 7 cm). Shortening

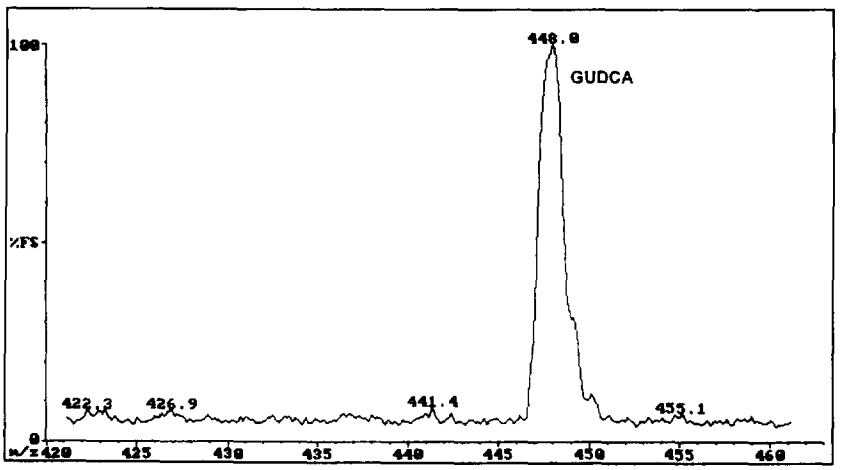

Fig. 2. Negative ion [M − H] spectrum of glycoursodeoxycholic acid obtained in the total-ion counting mode.

the column reduced the mobile phase flow-rate from 0.9 to 0.3 ml/min without altering the retention time or resolution, features which rendered it compatible with either the post-column splitter plus ES interface, or with the pre-column Acurate system.

The composition of the mobile phase developed for each class of BA (see above), was made to be compatible with the ES interface: all contained only volatile compounds at low concentrations and no adducts with acetate or ammonium ions were present. The same mobile phases were compatible for use with the ELSD II detector. Fig. 3 illustrates the separation efficiency of this system for a mixture of glycine- and taurine-conjugated BA (Fig. 3A) and free BA (Fig. 3B). The BAs were separated with both the ES-MS and the ELSD II, as shown on the top of each figure.

The amount of each BA standard injected with the Waters 717 autosampler was 2 μl (~1.5 ng). The Valco fixture split off 3% of the eluate into the ES interface system (45 pg); the remainder of the eluate was directed into the ELSD II detector; this arrangement explains the generation of a similar signal despite the differences in sensitivity of the two detectors (see Fig. 3). The results of the simultaneous analysis of both free and conjugated BA using the mobile phase gradient mode are reported in Fig. 4; all BAs were efficiently separated within 55 min and properly identified. The retention times obtained under isocratic conditions for glycine- and taurine-conjugated BA (solvent A), and for free BA (solvent B) are listed in Table 2. For all BAs studied, the detection limit (signal-to-noise ratio of 3) was ~15 pg injected at the ES interface, irrespective of whether the BA were free or conjugated, their retention time, or mobile phase composition (Table 2). In contrast, with the ELSD II the detection limit was a function of the latter two of these parameters, as previously reported [10], and it was much higher than that of the ES detector.

With the ES-MS system, quantitative analysis was easily performed because the detector response in this system is linearly related to the amount injected.

The peak areas correlated well with the amount injected, and the slope of the regression line was similar for all conjugated BAs, but was slightly lower for the free BA (Table 2). Individual BAs were thus directly identified since the MS spectra gave the m/z values of each BA as negative ions $[M-H]^-$.

With the developed mobile phase, 3α,7α,-3α,7β- or 3α,12α-dihydroxy BA epimers with the same m/z were efficiently separated, thus permitting their identification despite similar MS spectra.

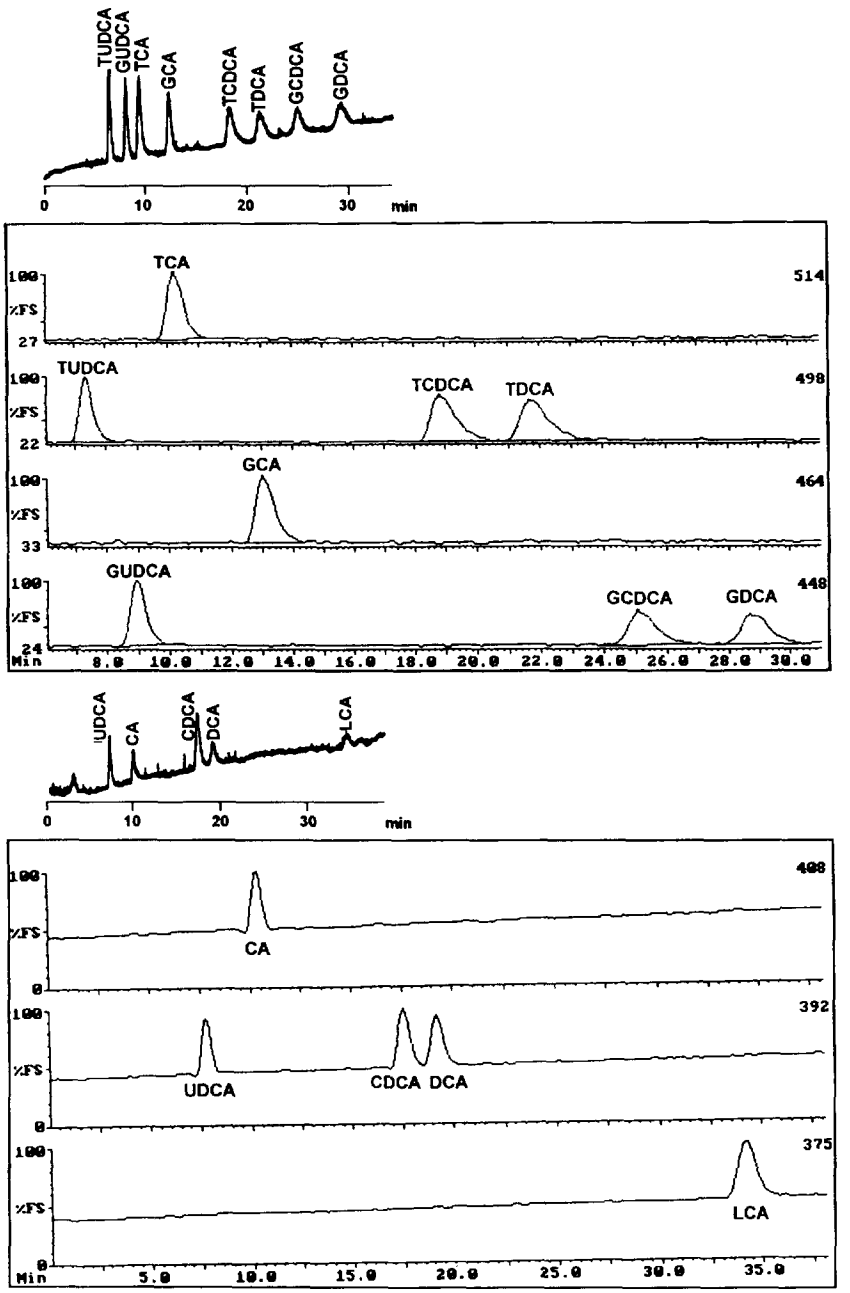

Fig. 3. Total-ion chromatograms obtained by HPLC–ES-MS analysis of bile acid standards (approx. 40 pg for each BA) using the conventional system. Separation of glycine- and taurine-conjugated BA with solvent A (see text) in isocratic chromatograms on the top of each figure were obtained with the evaporative light scattering mass detector on line with the ES-MS system. For abbreviations see text.

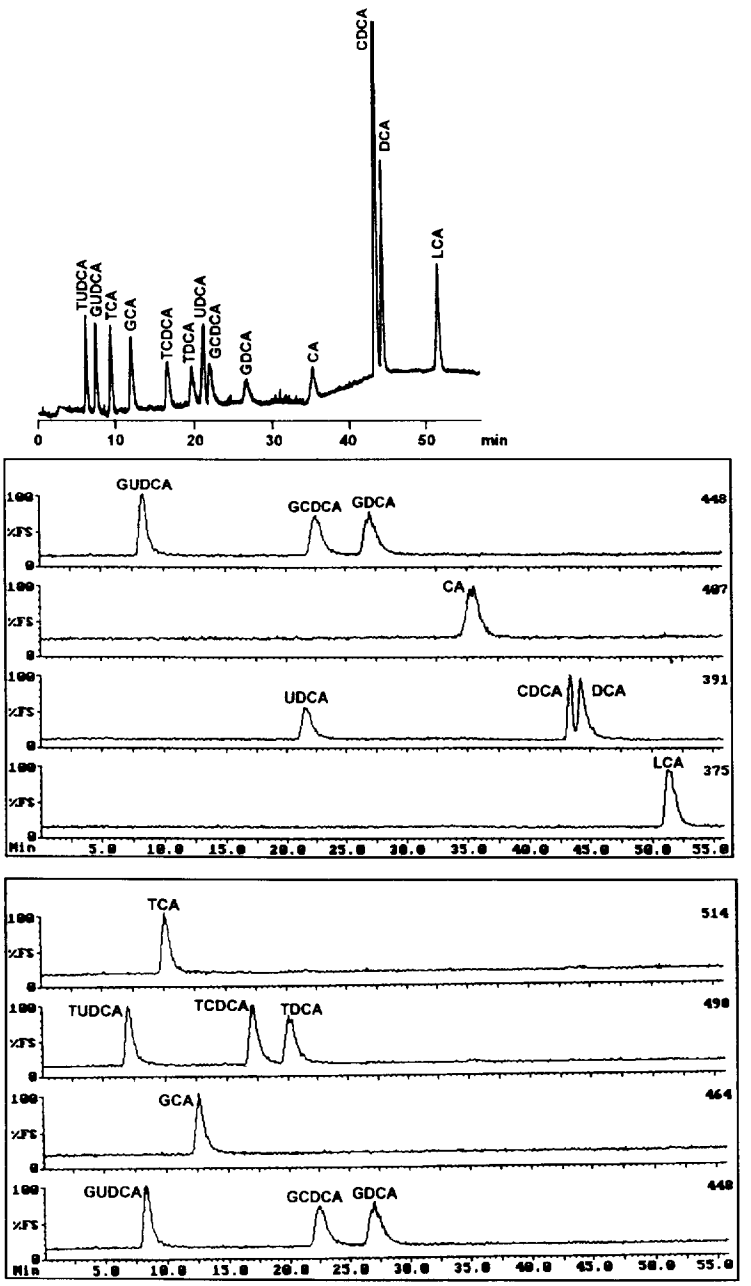

Fig. 4. Total-ion chromatograms from the injection of a mixture of bile acid standards (free, glycine- and taurine-conjugated) at the 40 pg level. Separation is achieved in gradient conditions (Table 1) using the conventional system. The top chromatogram is obtained with the evaporative light scattering mass detector on line with the ES-MS system. For abbreviations see text.

3.3. Semi-micro HPLC analysis

The Acurate system accurately reduced the initial mobile phase flow-rate from 300 to 1.4 μl/min, a rate which permitted use of a C_{18} microbore column Fusica™ with 0.30 mm I.D. Utilizing the same mobile phase as for conventional HPLC and 60 nl of the standards at a

Table 2
Retention time, detection limit and function of the calibration graphs of the studied BAs

BA	Retention time ES-MS (min)	Detection limit ES-MS (pg/inj.)	a	b	s_a	s_b	r
TUDCA	7.31	15	1.10	$2.29 \cdot 10^6$	0.15	$1.51 \cdot 10^5$	0.99
GUDCA	8.97	15	2.30	$2.80 \cdot 10^6$	0.28	$4.82 \cdot 10^5$	0.99
UDCA	7.75	15	3.20	$5.45 \cdot 10^5$	0.85	$6.83 \cdot 10^4$	0.98
TCA	10.21	15	1.70	$2.91 \cdot 10^6$	0.44	$2.90 \cdot 10^5$	1.00
GCA	13.05	15	4.42	$3.00 \cdot 10^6$	0.84	$2.11 \cdot 10^5$	0.99
CA	10.35	15	7.01	$4.52 \cdot 10^5$	1.10	$6.92 \cdot 10^4$	0.98
TCDCA	18.81	15	0.95	$2.60 \cdot 10^6$	0.40	$1.81 \cdot 10^5$	0.99
GCDCA	25.09	15	−0.14	$2.69 \cdot 10^6$	0.10	$2.12 \cdot 10^5$	0.99
CDCA	17.60	15	1.45	$6.92 \cdot 10^5$	0.40	$4.81 \cdot 10^4$	0.98
TDCA	21.73	15	2.82	$2.81 \cdot 10^6$	0.35	$1.71 \cdot 10^5$	0.99
GDCA	29.68	15	4.30	$2.80 \cdot 10^6$	1.10	$1.72 \cdot 10^5$	0.99
DCA	19.15	15	0.95	$8.05 \cdot 10^5$	0.20	$8.42 \cdot 10^4$	0.99
LCA	34.15	15	1.40	$6.90 \cdot 10^5$	0.35	$4.23 \cdot 10^4$	0.98

Retention time refers to solvent A for glycine and taurine conjugates and to solvent B for free BA under isocratic conditions. Equation of graph: $A = a + bx$ where A = peak area; x = pmol injected; a = intercept; b = slope; s_a = standard deviation of intercept; s_b = standard deviation of slope; r = correlation coefficient.

similar concentration, we obtained good separation of all BAs in a shorter time (16 min vs. 35 min, see Fig. 5). The detection limit of this system was lower than that of the conventional HPLC system; if the amount of BA injected rather than the amount reaching the detector is considered, the detection limit was 15 pg. The signal-to-noise ratio was also improved. The reproducibility of this system in terms of retention time and area of the eluted peaks was satisfactory under constant temperature conditions. The mobile phase flow-rate was ideal for the ES interface.

Analytical performance was enhanced by use of a loop injector of 200 nl and a head-enrichment technique: the BA standard to be injected was dissolved in methanol–water (20:80, v/v) instead of in the mobile phase; under these

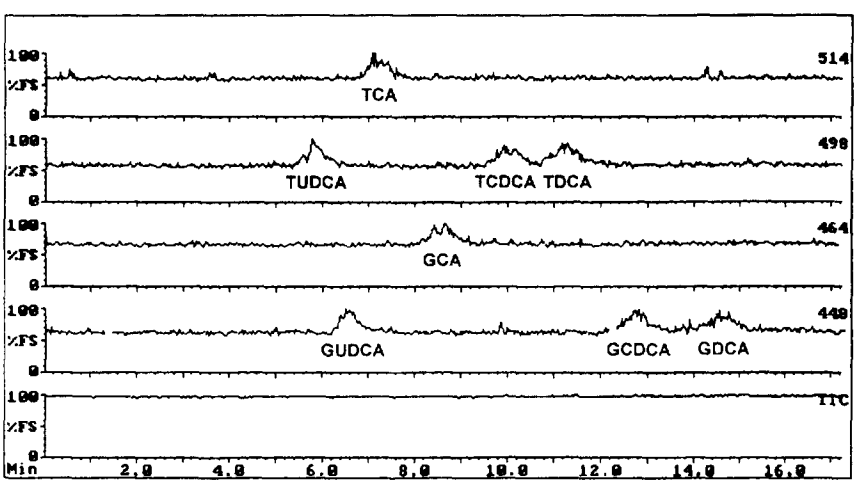

Fig. 5. Total-ion chromatogram obtained by microbore HPLC–ES-MS analysis of a mixture of eight BA standards, at a level of 20 pg per component.

conditions, the BAs were retained on the column and then eluted by the mobile phase in a very small volume. The final result was a relatively concentrated BA mixture that, being dissolved in a narrow volume, was efficiently resolved by the microcolumn and, in this way, the detection limit was lowered to 5 pg per injection. This system would be particularly useful for the analysis of BAs present in low concentration, as would be the case with serum or urine.

3.4. Applications

Biliary bile acid analysis

For chromatographic analysis, gallbladder bile (100–150 m*M*) is usually highly diluted (1:500–1:1000, (v/v)) with the mobile phase and only needs to be filtered (0.22-μm filter type GS, Millipore) before direct injection onto the HPLC column, with no other preliminary clean-up procedure being necessary. With the developed systems, individual BAs in bile can be easily separated and identified using less than 1 μl of sample.

As an example of the application of these systems to identification of an unknown BA, we analyzed the BA in gallbladder bile collected from hamsters treated with 3α,7β-dihydroxy-6-fluoro-5β-cholan-24 oic acid (6-FUDCA). This analog of UDCA was synthesized in our laboratory with the purpose to develop a drug that is more active than UDCA for the treatment of cholestatic liver disease. To determine the efficacy of this analog, all common BAs had to be separated and identified, which was accomplished with the ES-MS detector (Fig. 6). Simultaneous detection of the eluted BAs was performed with the ELSD II detector connected to the splitter.

This 6-fluoro UDCA analog is a mixture of 6α- and 6β-epimers that undergo hepatic amidation with glycine and taurine; the resulting hepatic metabolites, i.e. the glycine and taurine amidates of both epimers, can be found in the bile. These amidates and their epimers were identified by molecular mass *m/z* and retention times. Unknown dihydroxy and trihydroxy glycine- and taurine-conjugated BA were also found to be present; these compounds could not be properly identified without standards because their MS spectra were similar to those of other BAs, and also because there were no specific fragmentation products.

Serum bile acid analysis

Serum concentrations of individual BAs were determined from normal Syrian Golden hamsters whose total BA concentrations averaged 1.2 μM. Using a conventional system, BA can be detected with sufficient sensitivity with the ES-MS system, but not with the ELSD II detector, as shown in Fig. 7. The chromatograms in Fig. 8 show analysis of human serum from a patient with liver disease undergoing therapy with UDCA (total BA concentration was 50 μM).

As can be seen from our results, endogenous BAs such as GCA, TCDCA and GCDCA can be easily identified, and TUDCA and GUDCA, the hepatic metabolites of UDCA, can also be properly quantitated. With a potential detection limit for each BA of 15 pg injected, the HPLC–ES-MS analysis can be performed on less than 50 μl of serum, thus facilitating studies on small animals or BA kinetic studies which require frequent collection of small serum sample.

^{13}C stable isotope HPLC analysis

BAs labeled with stable isotopes such as ^{13}C or ^{15}N are powerful tools for studying BA metabolism and pharmacokinetics in animals and man, and have the additional advantage of not being radioactive.

Isotope dilution kinetic studies require an accurate calculation of the $^{13}C/^{12}C$ isotope ratio. This is usually achieved with a GC–MS system or with a specifically designed MS apparatus, i.e. one that has a window for the given isotope. We performed a preliminary study on the determination of the $^{13}C/^{12}C$ ratio in serum specimens after oral administration of glycoursodeoxycholic acid labeled with ^{13}C in the glycine moiety (carboxy group). Before HPLC analysis, calibration was done by analyzing several GUDCA standards enriched with ^{13}C-labeled GUDCA (0–10%, w/w). The isotope ratio in the GUDCA peak was determined by recording the

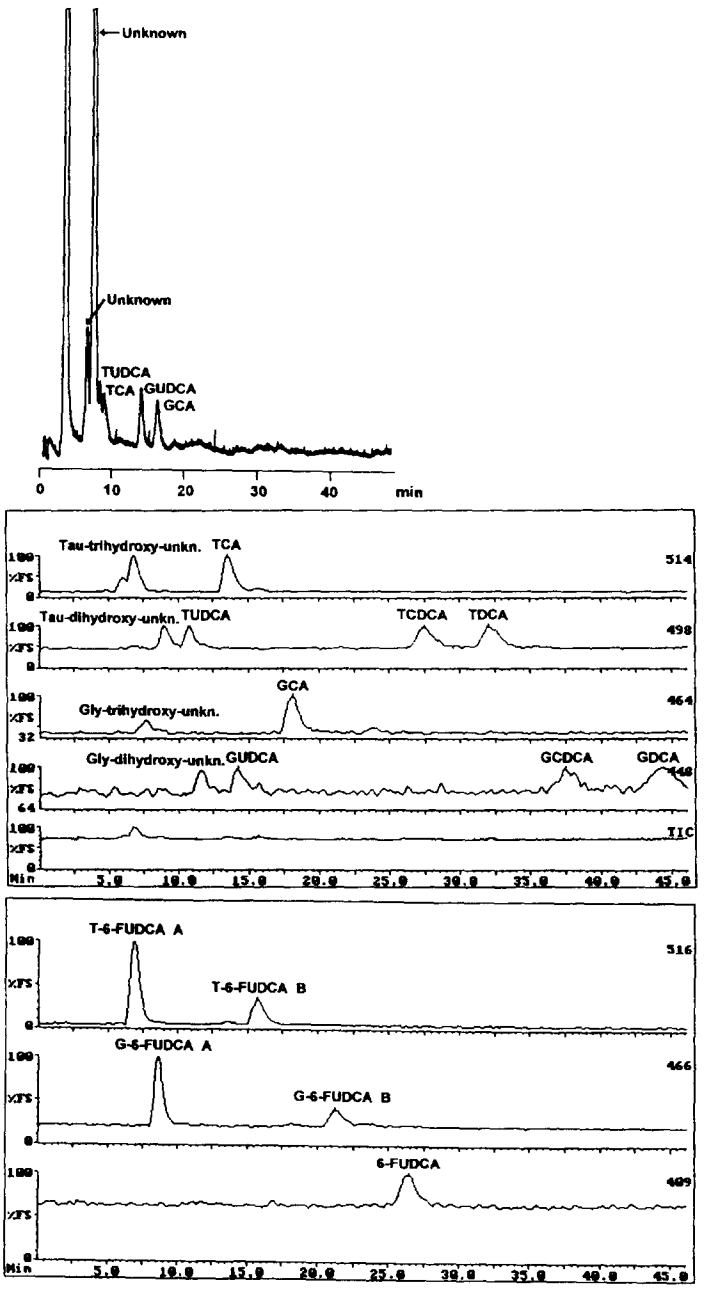

Fig. 6. Typical separation of common BA and 6-fluoro-3α,7β-dihydroxy-5β-choloic acid metabolites in hamster bile obtained with the HPLC–ES-MS system, using the conventional set-up.

$[M-H]^-$ selected ions of m/z 448 and 449, respectively (Fig. 9), and plotting the results against the calibration curve, which is given by the following equation: $y = 36.2133(\pm 0.423) + 0.374(\pm 0.069)x$. Even though resolution was not ideal, a good correlation was found, as demonstrated by an excellent regression line. These preliminary results indicate that this system has

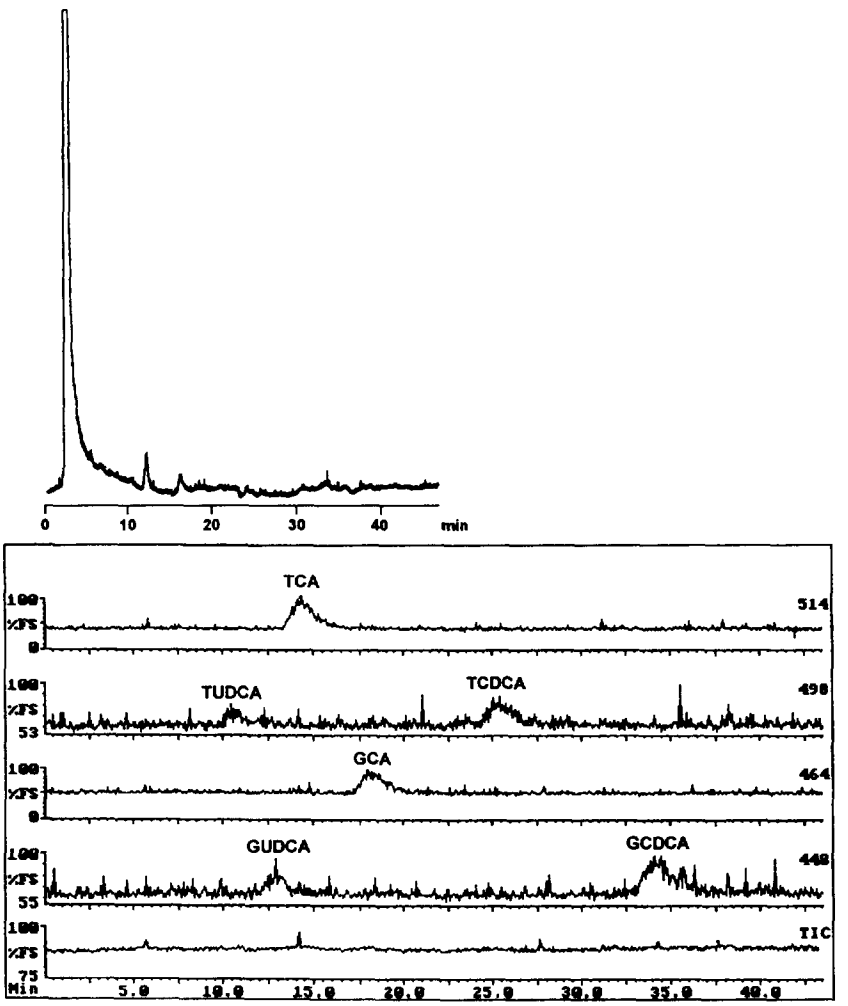

Fig. 7. Total-ion HPLC–ES-MS analysis of serum bile acids from a Syrian Golden hamster. The top chromatogram was obtained with the ELSD II detector on line. For abbreviations see text.

good potential for use in pharmacokinetic studies such as the determination of BA peaks by the isotope dilution method of individual amidated BAs.

4. Conclusions

This study has demonstrated the usefulness of the electrospray HPLC–MS technique for BA analysis. Coupled with either post-column splitting or Acurate semi-micro apparatus, the developed system exhibits a low detection limit, in the order of 10 pg per injection for each BA, which is lower than has been previously published for a thermospray interface. Good resolution of more than twelve BA from a given sample can be obtained in a short time, less than 20 min with the semi-micro system. Using the gradient system, all classes of BA including free, glycine- and taurine-conjugated forms can be analyzed in a single run, with a similar detection limit for all BAs of a given class. The sensitivity of this method is higher for glycine- and taurine-conjugated BAs than for free BAs, as shown by the slope of the calibration curves. The ES

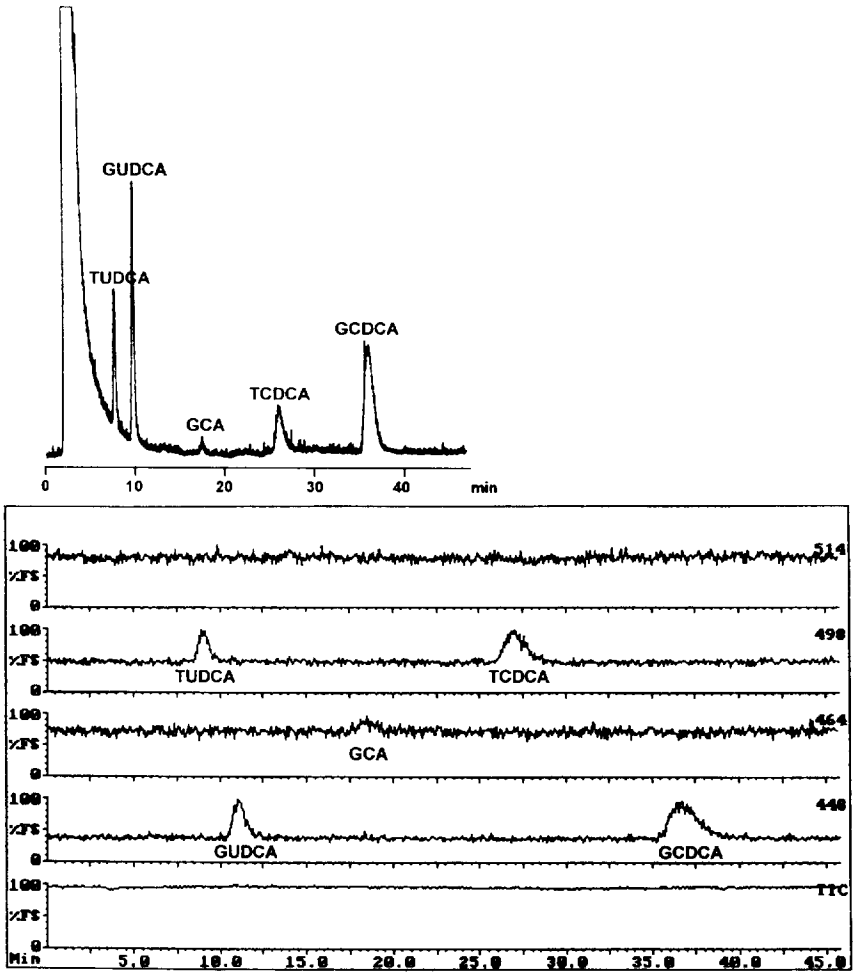

Fig. 8. Total-ion HPLC–ES-MS analysis of serum bile acids from a patient with cholestatic liver disease after oral therapy with UDCA. The top chromatogram was obtained with the ELSD II detector on line. For abbreviations see text.

generates $[M - H]^-$ ions without molecular fragments giving the molecular mass of the separated BA directly; the presence of fragments would be helpful for analyses requiring resolution of all BAs and their isomers.

The developed method is useful for BA analysis in bile in which direct measurements can be performed without any preanalytical step.

Serum BA concentration can also be measured; with the semi-micro system, less than 50 μl of serum are required for efficient and accurate analysis. This is an important technical improvement over previous direct HPLC methods which lack sensitivity and which can only be applied for serum BA determination after pre-column derivation procedures for the formation of fluorescent derivatives [11].

The ES interface is a relatively new system and, as such, needs further study, particularly with respect to the detection of the less common conjugated BAs such as sulfates, glucuronide ethers, or esters with multiple charges. The preliminary results obtained with the $^{13}C/^{12}C$ isotope ratio determinations demonstrate the promising potential that this system offers for isotope dilution studies in humans; it should allow better definition of BA metabolism, pharmacokinetics, and distribution of the BAs in the

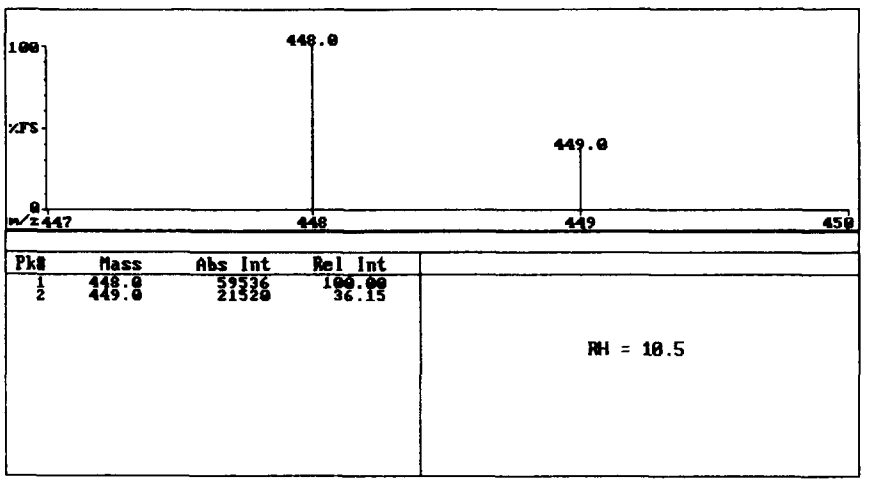

Fig. 9. HPLC–MS analysis of ^{13}C-labeled glycoursodeoxycholic acid showing the $^{13}C/^{12}C$ isotope ratio determination. ES-MS spectrum in selected ion monitoring mode: $[M-H]^- = 448$ and $[M-H]^- = 449$; $^{13}C/^{12}C$ ratio.

enterohepatic circulation. However, the system does require further improvement in terms of resolution of the negative ions.

In conclusion, HPLC–electrospray mass spectrometric analysis of BA is a promising technique that combines simple preanalytical steps, efficient resolution, high sensitivity, and a low detection limit. With this system, quantitative and qualitative analysis of BA can be performed on any biological fluid, including serum.

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References

[1] J. Goto, H. Kato, Y. Sarenta and T. Nambara, *J. Liq. Chromatogr.*, 3 (1980) 991.
[2] F. Nakayama and M. Nakagaki, *J. Chromatogr.*, 183 (1980) 287.
[3] A.T. Ruben and G.P. van Berge Henegouwen, *Clin. Chim. Acta*, 119 (1982) 41.
[4] N.P. Parris, *Anal. Biochem.*, 100 (1979) 260.
[5] S. Kamada, M. Maeda and A. Tsuji, *J. Chromatogr.*, 272 (1983) 29.
[6] J. Goto, M. Saito, T. Chikai, N. Goto and T. Nambara, *J. Chromatogr.*, 276 (1983) 289.
[7] G. Mingone, A.V. Greco and S. Passi, *J. Chromatogr.*, 183 (1980) 277.
[8] A. Hofmann and A. Roda, *J. Lipid Res.*, 25 (1984) 1477.
[9] A. Roda, A. Minutello, M.A. Angelotti and A. Fini, *J. Lipid Res.*, 31 (1990) 1433.
[10] A. Roda, C. Cerrè, P. Simoni, C. Polimeni, C. Vaccari and A. Pistillo, *J. Lipid Res.*, 33 (1992) 1393.
[11] A. Roda, R. Gatti, V. Cavrini, C. Cerrè and P. Simoni, *J. Pharm. Biomed. Anal.*, 11 (1993) 751.
[12] A. Stolyhwo, H. Colin and G. Guiochon, *J. Chromatogr.*, 265 (1983) 1.
[13] A. Stolyhwo, H. Colin, M. Martin and G. Guiochon, *J. Chromatogr.*, 288 (1984) 253.
[14] J. Sjövall, *Methods Biochem. Anal.*, 12 (1964) 97.
[15] T.J. Laatikainen, P.J. Lehtonen and A.E. Hesso, *J. Lab. Clin. Med.*, 19 (1978) 185.
[16] G. Karlaganis and G. Paumgartener, *J. Lipid Res.*, 19 (1978) 771.
[17] K.D.R. Setchell and A. Matsui, *Clin. Chim. Acta*, 127 (1983) 1.
[18] B. Almè, A. Bremmelgaard, J. Sjövall and P. Thomassen, *J. Lipid Res.*, 18 (1977) 339.
[19] D.R. Setchell and C.H. Vestal, *J. Lipid Res.*, 30 (1989) 1459.
[20] P. Kebarle and L. Tang, *Anal. Chem.*, 65 (1993) 972.
[21] T.R. Covey, E.D. Lee, A.P. Bruins and J.D. Henion, *Anal. Chem.*, 58 (1986) 1451.
[22] R.M. Caprioli, *Anal. Chem.*, 62 (1990) 477.
[23] T.G. Heath and A.B. Giordani, *J. Chromatogr.*, 638 (1993) 9.

[24] M.Y. Ye and Y. Shen, *J. Liq. Chromatogr.*, 17 (1994) 773.
[25] R.A. Newman, A. Fuentes, T.Y. Minor, K.T. McManus and D.A. Garteiz, *J. Liq. Chromatogr.*, 17 (1994) 403.
[26] J.F. Lawrence, B.P.Y. Lau, C. Cleroux and D. Lewis, *J. Chromatogr. A*, 659 (1994) 119.
[27] R.F. Straub and R.D. Voyksner, *J. Chromatogr.*, 647 (1993) 167.
[28] K.D.R. Setchell and J. Worthington, *Clin. Chim. Acta*, 141 (1982) 261.
[29] S. Scalia, *J. Pharm. Biomed. Anal.*, 3 (1990) 235.